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Activation of MAPK in fibroblasts by *Treponema denticola* major outer sheath protein

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Abstract

The major outer sheath protein (Msp) of *Treponema denticola* induces Ca^{2+} entry and actin reorganization in cultured fibroblasts, but the pathways by which Msp mediates these responses are not yet defined. We considered that Msp may activate protein kinases as a stress response that precedes actin remodelling. Phospho-kinase screens showed that Msp induced phosphorylation of multiple kinases in pathways that respond to extracellular agonists and regulate actin assembly. 34 kinases were significantly activated, including p38 and ERK 1/2. Accordingly, the expression and phosphorylation of p38 and ERK 1/2 in whole cell lysates were measured by immunoblotting and densitometry. Both kinases responded in a dose- and time-dependent manner to Msp exposure, were inhibited by SB202190 and U1026, respectively, and were unaffected by extracellular Ca^{2+}. These data indicate that *T. denticola* Msp may exert transient stress on fibroblasts through activation of MAP kinase pathways.

Keywords: *Treponema denticola*; Msp; Pore-forming toxin; MAPK phosphorylation; p38; ERK 1/2; Fibroblasts; Kinase proteome

Treponema denticola, an oral spirochete, is one of the key pathogens of periodontitis [1]. The bacterium, extracts of its outer membrane, and enriched preparations of its major outer sheath protein (Msp) can induce actin filament reorganization in cultured human gingival fibroblasts (HGFs) and Rat-2 fibroblasts [2–4]. These modifications lead to diminished attachment of fibroblasts to collagen, decreased cell spreading, and retarded migration [3,5]. Msp also induces acute cytoplasmic Ca^{2+} transients [6]. While there is some evidence that cytoplasmic Ca^{2+} transients are involved in the regulation of actin assembly [6], the signal transduction pathways that regulate Msp-induced alterations of actin filaments are still undefined.

Several types of protein kinases provide robust systems to protect cells from stressful stimuli. The Msp of *T. denticola* is a porin that can form ion-conducting channels in black lipid bilayers *in vitro* [7] and in the plasma membrane of cultured epithelial cells [8]. Since other bacterial pore-forming toxins can induce ion flux-responsive activation of mitogen-activated protein kinases (MAPK) [9], which in turn can regulate actin assembly [10,11], we asked whether Msp affects phosphorylation of protein kinases in fibroblasts. These proteins are important constituents of cell survival, transcription regulation, and cytoskeletal responses. We conducted a comprehensive survey of phospho-kinase activation and, based on these data, determined whether p38 and ERK 1/2 activation in fibroblasts was affected by Msp.

Materials and methods

Cell culture. Rat-2 fibroblasts were cultured in T-75 flasks at 37 °C in a CO_2-incubator in α-MEM with antibiotics and 10% heat-inactivated fetal bovine serum. For experiments, 1.6×10^5 cells (4 ml) were seeded in 60-mm dishes and incubated for 48 h at 37 °C. At 85–95% confluency, cells were washed with pre-warmed α-MEM prior to exposure to Msp diluted as described.

Human gingival fibroblasts (HGFs) were derived from primary explant cultures as described [12] and cells at passage 6–19 were cultured

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with 15% FBS and α-MEM. Dishes were seeded with 5×10^5 (4 ml) cells and incubated for 3 days prior to experiments.

Enrichment of Msp. Preparation of an enriched fraction of Msp from *T. denticola* ATCC 35405 was produced as previously described [6–8] and aliquots were kept frozen at −20 °C until used. Stock solutions were sonicated before use and diluted to specified concentrations in α-MEM or in a modified Hank's buffered salt solution (HBSS–HEPES) [13]: 150 mM NaCl, 5 mM KCl, 10 mM D-glucose, 1 mM $MgSO_4$, 1 mM Na_2PO_4, and 20 mM HEPES at pH 7.4. For experiments requiring Ca^{2+}-free buffer, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) was added at a final concentration of 5 mM.

Kinase screens. A kinase expression screen (KPKS-1.2) and a phospho-kinase screen (KPSS-8.0) were performed (Kinexus Bioinformatics Corporation, Vancouver, BC). Two separate sets of Msp- and vehicle-treated Rat-2 cell lysates pooled from 12 to 14 dishes for each condition were analyzed after stimulation by Msp, as optimized in pilot experiments based on p38 activation responses.

Impact of Msp on MAPK activation. Positive controls consisted of washed monolayers of cells stimulated with UV light (280 nm) or with thapsigargin (Sigma; 1 μg/ml). For experiments, cells were incubated with Msp (5, 10, 15, or 20 μg/ml) or with Msp-free vehicle for 5, 15, or 30 min. Cells were washed twice with PBS. Cold harvesting buffer (250 μl), containing 50 mM Tris–HCl at pH 7.5, 10 mM EDTA, 2 mM *o*-vanadate, 1 mM tetrasodium pyrophosphate, 20 mM β-glycerophosphate, 50 mM sodium fluoride, 5% Triton X-100 (final pH 7.4) supplemented with freshly diluted protease inhibitor cocktail (Sigma; 1/200), specific for the inhibition of serine, cysteine, aspartic proteases, and aminopeptidases, was added to each dish and incubated on ice for 15 min. The remaining cells and cell lysates were pooled and left on ice for another 10 min before centrifugation. Proteins in the supernatant were quantified by Bio-Rad (Mississauga, Ont.) protein assay using BSA as standard. Samples were diluted to the desired protein concentration and boiled in sample buffer for 5 min. The specificity of Msp in MAPK activation was verified by absorbing enriched Msp with anti-Msp antibodies and by heat-inactivation [5,6].

Immunoblotting. SDS–PAGE was performed at room temperature using the Laemmli buffer system [14], and the proteins were transferred to a nitrocellulose membrane (Bio-Rad; 0.45 μm). Specific primary antibodies for p38, pp38, ERK 1/2, and pERK 1/2 (Cell Signaling Technology, Denver, MA) and secondary antibody labeled with horse-radish peroxidase (Bio-Rad) were used as directed by the manufacturer and detected by chemiluminescence (Amersham Biosciences, Baie d'Urfé, Qué.). Gels were analyzed with ImageJ [15].

Actin filament labeling. Cells were seeded at 2×10^5 cells/ml and grown for two days to 60% confluency. Actin filaments were labeled with Alexa Fluor 488 phalloidin (Molecular Probes, Burlington, Ont.) [3]. The cells were examined by fluorescence microscopy at 495/518 nm wavelengths (E_x/E_m). Images were recorded with SPOT Advance software.

Statistical analysis. Statistical analysis for group comparisons was done using Student's *t*-test. Means ± standard errors were calculated. Differences were considered significant at $p < 0.05$.

Results and discussion

Analysis of kinase expression and phosphorylation profiles

Two kinase screens were used to analyze kinase responses of Rat-2 fibroblasts exposed to Msp. The first screen estimated the expression levels of 75 kinases (KPKS-1.2, Kinetworks™ Protein Kinase Screen 1.2); 56 of these kinases were detected in samples from control- and Msp-treated cells. p38, ERK 1/2 and JNK were among the 36 kinases with <25% difference between Msp-treated and control-treated cells (Table 1). The second screen estimated the activation profile of selected stress kinases (KPSS-8.0, Kinetworks™ Phospho-Site Stress Screen 8.0) by measuring the phosphorylation levels of 34 kinases in lysates of Msp- and vehicle-treated fibroblasts by phospho-specific antibodies and immunoblotting. Elevated phosphorylation levels of p38, ERK 1/2, and JNK (Table 1) indicated that three major pathways commonly activated by stressful conditions were also activated by Msp.

A number of kinases that are linked to stress responses can also regulate actin assembly, and these were generally affected by Msp (Table 1). JNK, which is normally activated by PAK and PAK 1/2/3, exhibited increased phosphorylation in Msp-treated cells. Upstream to PAK 1/2/3, the small GTPases Rac and cdc42 also showed increased phosphorylation after exposure to Msp. Phosphorylation of PAK 1/2/3 and Rac/cdc42 can induce phosphorylation of cofilin, a key actin-severing protein [16,17]. Our findings were consistent with these previous reports since cofilin phosphorylation was increased in Msp-treated cells. Two other proteins that are downstream of p38 and linked to actin assembly, heat shock protein (Hsp) 27 and Hsp25, were found to be phosphorylated after treatment and may be implicated in the actin filament reorganization caused by Msp. Many bacterial toxins induce cytoskeletal reorganization [18,19] including pertussis toxin (PTX), which mediates p38 and Hsp27-dependent cytoskeletal rearrangement in endothelial cells [20]. Hsp27 is associated with actin polymerization, but it also has antiapoptotic activity [21]. Perhaps Hsp27 activation may foster the reported maintenance of fibroblast viability upon Msp exposure [5,6,22].

Activation of p38 and ERK 1/2 is Msp time- and concentration-dependent

Based on the findings from the phospho-kinase screen that showed strong increases of phosphorylation after Msp treatment for all kinases measured with this assay, we focused on p38 and ERK 1/2. Phosphorylation of both p38 and ERK 1/2 was time-dependent for Rat-2 cells and the response was maximal after 15 min of treatment ($p < 0.05$; Figs. 1A and 2A). There was no significant change in the expression of p38 protein in response to Msp. Phosphorylation of p38 was increased in a concentration-dependent manner in response to Msp ($p < 0.05$; Fig. 1C). Similarly, there was no change in the ERK 1/2 protein content, and a similar concentration-dependent increase of phosphorylated ERK 1/2 was observed with a peak response at 10 μg/ml of Msp, followed by a plateau (Fig. 2C).

Identical experimental designs were conducted with human gingival fibroblasts (HGFs) (Figs. 1B, D and 2B, D). Similar to results for Rat-2 cells, the expression level of p38 and ERK 1/2 was unchanged by Msp treatment and there was a robust increase in phosphorylation of p38 and ERK 1/2 with time and concentration ($p < 0.05$). However, a clear-cut concentration and peak exposure time were not as clearly evident with Rat-2 cells in comparison to human gingival fibroblasts (Figs. 1B and 2D).

Table 1
Protein kinase expression and phosphorylation level[a] in Rat-2 fibroblasts

Full name of protein	Abbreviation	% Change of expression[b]	% Change of phosphorylation[b]
Cofilin 1	Cofilin 1	NT	1106
Extracellular regulated protein-serine kinase 1	Erk1	−13	542
Extra cellular regulated protein-serine kinase 2	Erk2	−9	290
Heat shock 25 kDa protein (mouse)	Hsp25 mouse	NT	608
Heat shock 27 kDa protein β1	Hsp27	NT	213
Jun N-terminus protein-serine kinase (37)	JNK	17	Increase
Jun N-terminus protein-serine kinase (46)	JNK	−7	Increase
Jun proto-oncogene-encoded AP1 transcription factor (41)	Jun	NT	327
Jun proto-oncogene-encoded AP1 transcription factor (39)	Jun	NT	Increase
Mitogen-activated protein-serine kinase p38α (36)	p38a MAPK	1	1954
p21-activated protein-serine kinase 1/2/3	PAK1/2/3	NT	449
Protein-serine kinase B α (Akt1)	PKBa (Akt1)	NT	Increase
Ras-related C3 botulinum toxin substrate 1	Rac1/cdc42	NT	1433
Signal transducer and activator of transcription 3	STAT3	NT	263

NT, not tested.
Increase: the phosphorylated form was not detected in the control but was present in Msp-treated cells.
[a] Kinetworks™ Protein Kinase Phosphorylation Screen, Kinexus Corporation, Vancouver, BC, Canada.
[b] Significant change defined by Kinexus as >25% increase or decrease compared with values for cells treated with vehicle control.

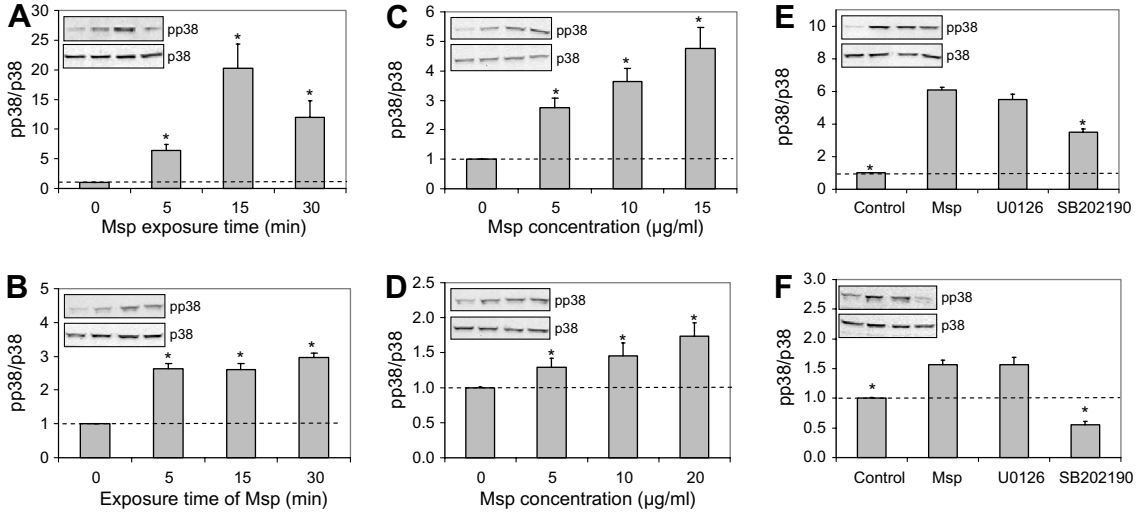

Fig. 1. Activation of p38 in Rat-2 fibroblasts and HGFs challenged with Msp. Cell monolayers were exposed to Msp at different times and concentrations. Data bars represent means ± standard error of the ratio of activated to total p38 as determined by densitometry ($n = 3$ independent experiments, in duplicate). A dotted line was traced at 1.0, which was the ratio of the basal level (control) to which every other condition was normalized. (Inset) Typical Western blot from one experiment. *, significant difference from control ($p < 0.05$). (A) Time-course with Msp (20 μg/ml) in Rat-2 cells; (B) HGFs. (C) Dose–response for exposure to Msp at specified concentrations for 15 min in Rat-2 cells; (D) HGFs. (E) Rat-2 cells pre-incubated for 30 min with p38 inhibitor (SB202190; 10 μM in 0.1% DMSO, IC_{50} 16 nM) or ERK 1/2 inhibitor (U0126; 10 μM in 0.1% DMSO, IC_{50} 72 nM). Medium was replaced by Msp (10 μg/ml) in fresh medium containing inhibitor for 15 min; (F) HGFs.

We asked whether p38 and ERK 1/2 were activated independently of one another. Two inhibitors, SB202190 and U0126, were chosen for their specific inhibition of p38 and ERK 1/2, respectively [23]. These molecules prevent the phosphorylation of the MAPK in a non-competitive and irreversible manner, diminishing the probability of interference between the pathways. DMSO-treated cells labeled with Alexa Fluor 488 phalloidin confirmed their integrity over the course of the experiment (shown in Supplement 1). Both kinases were inhibited in Rat-2 cells (51 ± 4% for p38 and 115 ± 28% for ERK) ($p < 0.05$, Figs. 1E and 2E). The data for HGFs were similar to those for Rat-2 cells (Figs. 1F and 2F). These results indicated that p38 and ERK 1/2 activation by Msp could be inhibited with minimal cross-interference and that activation of both p38 and ERK 1/2 probably involved distinct activation pathways.

Msp was enriched from a bacterial extract. As we had shown previously for Msp-induced actin perturbation and Ca^{2+} flux [5,6], absorption of the enriched Msp with anti-Msp antibodies, followed by binding to protein A and G agarose beads, reduced the activation of p38 and ERK 1/2 by 84 ± 4% and 93 ± 5%, respectively ($p < 0.05$; shown in Supplement 2), indicating that the active component was indeed the authentic Msp. Immunoblots of the absorbed Msp extract showed that less than 20% of the

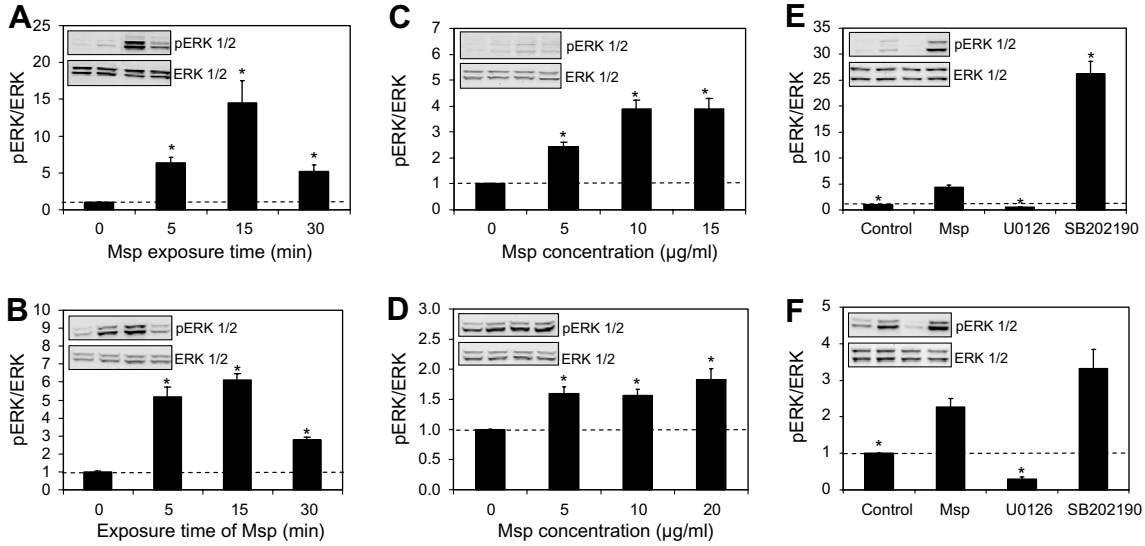

Fig. 2. Activation of ERK 1/2 in Rat-2 fibroblasts and HGFs challenged with Msp. Cell monolayers were exposed to Msp at different times and concentrations. Data bars represent means ± standard error of the ratio of activated to total ERK 1/2 as determined by densitometry ($n = 3$ independent experiments, in duplicate). A dotted line was traced at 1.0, which was the basal level to which every other condition was normalized. (Inset) Typical Western blot from one experiment. *, significant difference from control ($p < 0.05$). (A) Time-course with Msp (20 μg/ml) in Rat-2 cells; (B) HGFs. (C) Dose–response for exposure to Msp at specified concentrations for 15 min in Rat-2 cells; (D) HGFs. (E) Rat-2 cells pre-incubated for 30 min with p38 inhibitor (SB202190; 10 μM in 0.1% DMSO) or ERK 1/2 inhibitor (U0126; 10 μM in 0.1% DMSO). Medium with inhibitor was replaced by Msp (10 μg/ml) in fresh medium containing inhibitor for 15 min; (F) HGFs.

immunoreactive Msp was still present in the extract (shown in Supplement 3). Heating for 30 min or boiling Msp for 10 min also significantly reduced the activation of p38 by more than 50% ($p < 0.05$; shown in Supplement 2). Yet, heating had no effect on the activation of ERK 1/2. Apparently, the Msp domains responsible for ERK 1/2 activation are heat-stable and might be different than those inducing the phosphorylation of p38.

Our protein screens and Western blot analysis found that exposure of fibroblasts to Msp invariably activated p38 and ERK 1/2 without changing their total expression. Similar observations were reported by Leung and coworkers in epithelial cells exposed to a dense bacterial suspension of *T. denticola* [24]. Conceivably, Msp could contribute to MAPK activation by the whole bacterium. However, phosphorylation of p38 and ERK 1/2 seemed to activate apoptosis or proliferation, respectively, in epithelial cells [24], which we have not observed with Msp-treated fibroblasts [5,6,22]. Fibroblasts are evidently more resistant than epithelial cells to Msp-induced cell death [22].

The ability of Msp to form transient pores in the plasma membrane may be sufficient to induce cell stress responses. Enriched Msp forms conductive ion channels in HeLa cells [8]. Recently, epithelial cells were shown to respond by the activation of p38 to sub-cytolytic concentrations of several bacterial pore-forming toxins such as Ply, a toxin produced by *Streptococcus pneumoniae* [25]. p38 was phosphorylated independently of the recognition of Ply by toll-like receptor 4 (TLR4), and intracellular Ca^{2+} fluxes provoked by the entry of Ca^{2+} through the pore [25]. The formation of

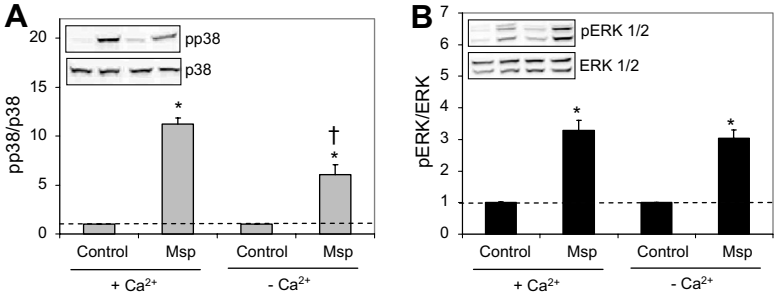

Fig. 3. Activation of p38 and ERK 1/2 in Ca^{2+}-containing and Ca^{2+}-free buffer in Rat-2 fibroblasts. Cell monolayers were exposed to a fresh dilution of Msp (10 μg/ml) with and without Ca^{2+} for 15 min after pre-treatment of 10 min of the monolayer with the chosen buffer. Data bars represent means ± standard error of the ratio of activated to total p38 or ERK 1/2 as determined by densitometry ($n = 3$ independent experiments, in duplicate). A dotted line was traced at 1.0, which was the basal level to which every other condition was normalized. (Inset) Typical Western blot from one experiment. Both (A) p38 and (B) ERK 1/2 were measured. *, significant differences ($p < 0.05$) of Msp treatment with its proper control. †, significant difference ($p < 0.05$) between Msp-treated in Ca^{2+}-free buffer and Msp-treated in Ca^{2+}-containing buffer.

pores by Msp in fibroblast membranes is plausible, since exposure of these cells to Msp causes acute influx of Ca^{2+} and Mn^{2+} [6].

Activation of p38 and ERK 1/2 by Msp is independent of calcium influx

Since Ca^{2+} is a ubiquitous messenger that can regulate MAPKs, we asked whether the activation of p38 and ERK 1/2 may result from Msp-induced Ca^{2+} influx. We have previously established that Msp-induced acute Ca^{2+} transients are due to the influx of extracellular Ca^{2+}, not by release of Ca^{2+} from internal stores [6]. Both p38 and ERK 1/2 were activated when exposed to Msp in both Ca^{2+}-containing and Ca^{2+}-free buffers (Fig. 3A and B). These conditions did not affect cell adherence or shape over the 25 min period (10 min pre-incubation and 15 min stimulation; shown in Supplement 4). Our data seem to be analogous to those reported for the Ca^{2+}-independent activation of p38 in epithelial cells by Ply [25]. While the mechanisms by which Msp acts extracellularly to dysregulate actin filament assembly are unclear, the activation of MAPKs known to be involved in receptor-mediated responses to extracellular agonists and toxic stress is apparently a common theme in microbial toxicity [26].

In summary, consistent with the activity of other pore-forming bacterial toxins [25], *T. denticola* Msp induces the activation of MAP and other kinases that mediate host cell responses to extracellular stressors. The time course for MAPK activation places it upstream of significant actin filament reorganization. Therefore, the activated kinase and cytoskeletal responses may be mechanisms by which fibroblasts protect themselves and remain viable in the presence of a bacterial protein that perturbs the plasma membrane.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.111.

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